Hypoglycemia attenuates acute amylin-induced reduction of food intake in male rats

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Abstract: The ability of amylin to inhibit gastric emptying and glucagon secretion in rats is reduced under hypoglycemic conditions. These effects are considered part of a fail-safe mechanism that prevents amylin from further decreasing nutrient supply when blood glucose levels are low. Because these actions and amylin-induced satiation are mediated by the area postrema (AP), it is plausible that these phenomena are based on the co-sensitivity of AP neurons to amylin and glucose. Using hyperinsulinemic glucose clamps in unrestrained and freely-feeding rats, we investigated whether amylin's ability to inhibit food intake is also reduced by hypoglycemia (HYPO). Following an 18 h fast, rats were infused with insulin and glucose for 45 min to clamp blood glucose at baseline levels (between 90 and 100 mg/dL). HYPO (approximately 55 mg/dL) was induced between 45 and 60 min and then maintained for the remainder of the clamp. Rats were injected with amylin (20 µg/kg) or saline and offered normal chow at 85 min. Food intake was measured at 30 and 60 min after amylin. Control hyperinsulinemic/euglycemic (EU) rats were maintained at approximately 150 mg/dL (which is a physiological periprandial glucose level) before and after amylin injection. Terminal experiments tested the effect of amylin to induce the phosphorylation of ERK, a marker of amylin action in the AP, in EU and HYPO conditions. Amylin significantly reduced 30- and 60-min food intake in EU rats, but the effect at 60-min was attenuated in HYPO rats. Interestingly, glucose infusion rate had to be dramatically reduced at meal onset in saline-treated, but not in amylin-treated, EU or HYPO rats; this suggests that meal-related glucose appearance in the blood was inhibited by amylin under both EU and HYPO. Finally, amylin induced a similar pERK response in the AP in EU and HYPO rats. We conclude that amylin's action to decrease eating is blunted in hypoglycemia, and this effect seems to be downstream from amylin-induced pERK in AP neurons. These data allow us to extend the idea of a hypoglycemic brake on amylin's actions to its food intake-reducing effect, but also demonstrate that amylin can buffer meal-induced glucose appearance at EU and HYPO levels.

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Hypoglycemia attenuates acute amylin-induced reduction of food intake in male rats

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ABSTRACT

The ability of amylin to inhibit gastric emptying and glucagon secretion in rats is reduced under hypoglycemic conditions. These effects are considered part of a fail-safe mechanism that prevents amylin from further decreasing nutrient supply when blood glucose levels are low. Because these actions and amylin-induced satiation are mediated by the area postrema (AP), it is plausible that these phenomena are based on the co-sensitivity of AP neurons to amylin and glucose. Using hyperinsulinemic glucose clamps in unrestrained and freely-feeding rats, we investigated whether amylin's ability to inhibit food intake is also reduced by hypoglycemia (HYPO). Following an 18 h fast, rats were infused with insulin and glucose for 45 min to clamp blood glucose at baseline levels (between 90 and 100 mg/dL). HYPO (approximately 55 mg/dL) was induced between 45 and 60 min and then maintained for the remainder of the clamp. Rats were injected with amylin (20 μg/kg) or saline and offered normal chow at 85 min. Food intake was measured at 30 and 60 min after amylin. Control hyperinsulinemic/euglycemic (EU) rats were maintained at approximately 150 mg/dL (which is a physiological periprandial glucose level) before and after amylin injection. Terminal experiments tested the effect of amylin to induce the phosphorylation of ERK, a marker of amylin action in the AP, in EU and HYPO conditions. Amylin significantly reduced 30- and 60-min food intake in EU rats, but the effect at 60-min was attenuated in HYPO rats. Interestingly, glucose infusion rate had to be dramatically reduced at meal onset in saline-treated, but not in amylin-treated, EU or HYPO rats; this suggests that meal-related glucose appearance in the blood was inhibited by amylin under both EU and HYPO. Finally, amylin induced a similar pERK response in the AP in EU and HYPO rats. We conclude that amylin's action to decrease eating is blunted in hypoglycemia, and this effect seems to be downstream from amylin-induced pERK in AP neurons. These data allow us to extend the idea of a hypoglycemic brake on amylin's actions to its food intake-reducing effect, but also demonstrate that amylin can buffer meal-induced glucose appearance at EU and HYPO levels.

1. Introduction

The mammalian body has several remarkable and intricate mechanisms to keep the organism at equilibrium. The interplay among hormones released from the pancreas represent one such mechanism for maintaining blood glucose. Insulin released from the pancreatic beta cells regulates glucose uptake from the blood, and glucagon from the alpha cells promotes utilization of endogenous glucose stores and gluconeogenesis when blood glucose is low. Amylin is a second beta-cell hormone with actions relevant for the control of nutrient metabolism, interacting with both insulin and glucagon. In response to nutrient stimuli, amylin is co-secreted with insulin and complements insulin action by limiting the rate of glucose appearance into the blood. Amylin achieves this through three major actions: the inhibition of postprandial glucagon secretion, slowing gastric emptying and by inducing satiation. This collectively demonstrates that amylin has important glycemic buffering properties that prevent excessive flooding of glucose into the blood, particularly during the prandial and postprandial phases (for review, see [1,2]).

Because of these properties, soon after its discovery, amylin was considered an interesting candidate to supplement insulin therapy in people suffering from type 1 and late-stage type 2 diabetes, and the amylin analogue pramlintide was eventually approved for use by the FDA. However, an early concern was whether amylin would be

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detrimental if a person requiring insulin had a hypoglycemic episode—increased glucagon secretion, accelerated gastric emptying and nutrient ingestion are critical measures for restoring glucose to healthy levels, measures which could inadvertently be blocked by amylin. Interestingly, it was found that under hypoglycemic conditions (35—65 mg/dL), amylin neither blocked glucagon release nor slowed gastric emptying. The presence of a protective hypoglycemic brake was suggested, which seems to prevent amylin-induced inhibitions of gastric emptying and glucagon secretion when blood glucose levels are critically low [3-5]. However, whether this protective mechanism blocks all glucose buffering of amylin, including its effect on satiation remained to be investigated. Furthermore, the mechanism underlying this brake has not been determined. The area postrema (AP) is the primary brain site mediating amylin's effects. Peripheral administration of amylin leads to increases in various markers of neuronal activation in the AP, including the phosphorylation of ERK (pERK) [6], and lesions of the AP reduce amylin's ability to reduce food intake [7], suppress glucagon and slow gastric emptying [8]. In addition to responding to amylin, the vast majority of these AP neurons are also glucose responsive; their rate of spontaneous activity decreases with decreasing glucose concentrations [9]. This has led to the suggestion that a certain level of glucose may be necessary or permissive of amylin action in the AP. Along this line of reasoning, low glucose levels may de-sensitize AP neurons to stimulation by amylin.

The primary goal of this study was to test the hypothesis that hypoglycemia limits the acute inhibitory effect of amylin on eating, in line with what was observed for glucagon release and gastric emptying. Secondly, we wanted to determine if glucose levels directly influence the ability of amylin to activate downstream signaling pathways, such as the phosphorylation of ERK, which seems to be functionally relevant for amylin action [6], in AP neurons. To test these hypotheses, we developed a novel hyperinsulinemic glucose clamp that allowed freely-moving rats to spontaneously eat while glycemic levels were maintained at either a euglycemic (EU) or hypoglycemia (HYPO) target. EU was defined as the physiological level of glycemia in postprandial rats.

2. Methods

2.1. Subjects

Male Wistar rats (200—225 g body weight on arrival) were purchased from Janvier, France. Rats were individually-housed in hanging, stainless steel wire-mesh cages. All cages were furnished with a plastic tunnel and nest-building material. Rats were maintained in a temperature-controlled environment (21± 2 °C), on a 12 h/12 h light-dark cycle. Water and standard chow (D3430; Provimi Kliba AG, Kaiseraugst, Switzerland) were accessible ad libitum, unless otherwise indicated. All experiments were performed with the approval of the veterinary office of the Canton Zurich, Switzerland, and in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Surgical preparation for hyperinsulinemic/eu- and hypoglycemic clamps

Chronic catheters were implanted in the carotid artery for frequent and stress-free blood sampling, and in the jugular vein for peripheral infusion of insulin and glucose during glycemic clamps. Approximately one week prior to experiments, rats were food-deprived overnight and received subcutaneous injections of antibiotics (enrofloxacin, 10 mg/kg) and analgesics (meloxicam, 2.5 mg/kg) prior to surgery. Rats were anesthetized using ketamine (80 mg/kg, i.p.; Narketan10) and xylazine (6 mg/kg, i.p.; Rompun 2%). Incision sites were shaved and disinfected with Betadine, and Vitamin-A ointment was applied to the eyes for protection. During surgery, rats received oxygen via nose cone (1.4 L/min), and body temperature was monitored and maintained with a rectal probe that automatically adjusted the temperature of a heating pad below the rat. Catheters (Silastic ID 0.51 x OD 0.94 mm; Instech Solomon, Plymouth Meeting, USA) were implanted in the carotid artery and in the jugular vein, and fixed to a mesh connector at the site between the scapulae where they were exteriorized. Blood flow was confirmed in both lines, and catheters were sealed with an autoclaved lock solution (100 IU/mL insulin in 50% glycerol) until the day of experiment. Rats were placed in a warmed chamber to recover from anesthesia, and received a post-operative dose of Flunixin (1 mg/kg, s.c.) and 5 ml of warm saline supplemented with 5% glucose (s.c.). Rats continued to receive subcutaneous injections of antibiotics and analgesics (dosing as above), and 0.9% saline supplemented with 5% glucose daily for the first 1 to 3 days after surgery.

2.3. Establishing a glucose clamp paradigm in freely-feeding rats

As glycemic clamps are typically performed in anesthetized or fasted rodents, we executed several pilot studies to establish three critical components of our new clamp paradigm that was performed in freely-feeding rats.

2.3.1. Determine glucose curves during a meal to establish euglycemic clamp target

The first pilot was performed to determine basal (after 18 h of fasting) and postprandial glucose levels in rats. Rats ($n = 3$) were prepared for the clamp procedure, however only saline was infused through the jugular vein catheter. At time point 0, fasted blood glucose was measured in arterial blood (82.8 ± 7.2 mg/dL), after which rats were given access to standard chow and immediately began to eat. Glucose was measured every 5 min for the first 5 time points, and every 10 min thereafter; food intake was measured at 30 and 60 min. As shown in Fig. 1, glucose levels already began to rise after 5 min, and peaked at 30 min from meal onset, reaching 156.8 ± 9.0 mg/dL. From there, glucose levels gradually decreased to 133.3 ± 1.8 mg/dL at 60 min after meal onset. Rats consumed 5.7 ± 0.3 g of food at 30 min, and 6.8 ± 0.6 g at 60 min, which is similar to intake in unmanipulated rats [7, 10]. Based on these findings, our target for physiological EU during the hyperinsulinemic/EU clamps was 150 mg/dL.

2.3.2. Determine infusion rates of insulin

We next established the insulin infusion rate necessary to maintain hypoglycemia in freely-feeding rats. We found that a typical insulin infusion rate of 25 mU/kg/min [11] was insufficient to maintain hypoglycemia when test rats ($n = 2$) began eating. To maintain a glucose level of approximately 55 mg/dL, it was necessary to increase the insulin infusion rate by approximately 4-fold. We therefore retested rats using 125 mU/kg/min insulin, which was sufficient to maintain a state of hypoglycemia in freely feeding rats. Based on these findings, all studies were performed using an insulin infusion rate of 125 mU/kg/min, which enabled constant insulin infusion conditions for the duration of each clamp. In order to account for this high insulin infusion rate during the fasted portions of the clamps, a 50% glucose solution was infused for the duration of each clamp.

2.3.3. Determine glucose infusion rate (GIR) adjustment after amylin

Finally, we observed during our pilot tests the importance of adjusting GIR, particularly after amylin treatment. We noted that in rats receiving saline injection, in order to maintain the target glycemic level, it was necessary to reduce their GIR to account for the meal-derived glucose. In rats maintained in a hypoglycemic state, it was often necessary to reduce the GIR to 0 mg/kg/min. However, if this same reduction was performed in rats receiving amylin prior to the meal, their glucose levels fell below our hypoglycemic target, and in some cases below 35 mg/dL, which ultimately affected their fine-motor coordination and ability to eat. Consequently, we found it necessary to increase

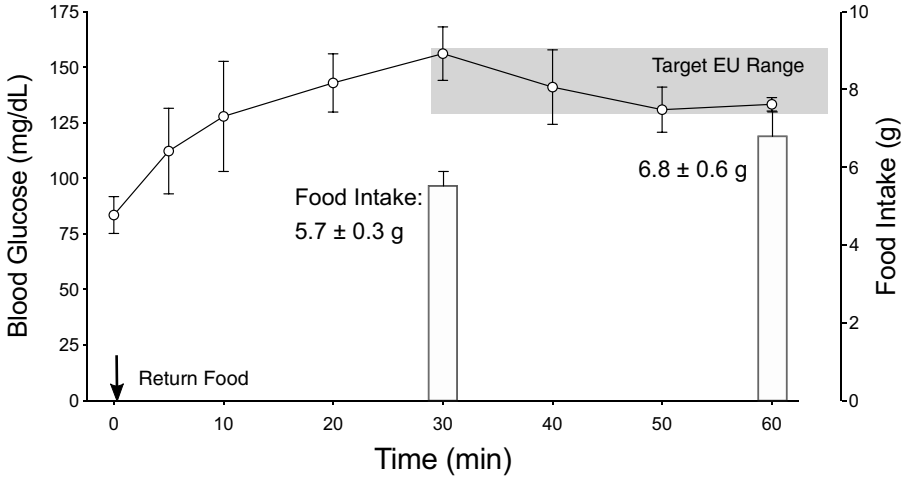

Fig. 1. Determination of postprandial glycemic levels. Mean ± SD arterial glucose levels and food intake measured in previously fasted rats ($n = 3$) for 60 min. Rats were attached to a weighted swivel tether and saline was infused via the jugular vein catheter for 60 min prior to food access. At timepoint 0 min, chow was given to the rats and blood glucose was measured via the carotid artery catheter every 5 to 10 min. A peak glucose level of 156.8 ± 9.0 mg/dL was reached 30 min after meal initiation, and by 60 min glucose levels decreased to 133.3 ± 1.8 mg/dL. This range therefore represented our target for the hyperinsulinemia-EU clamps.

the GIR in rats receiving amylin treatment, to prevent a drop in glucose level and thus preserving the rats' ability to eat during the clamp. We also observed that 55 mg/dL was a HYPO level at which rats maintained the fine-motor skills to eat normally.

2.4. Amylin action during hyperinsulinemic euglycemic and hypoglycemic clamps

A schematic timeline of the clamp experiments is shown in Fig. 2. Rats were fasted 18 h before the start of the experiment and placed in Macrolon cages with bedding for infusion. One hour before the start of the clamp, rats were connected to a tethered dual-channel swivel system (Instech Solomon, Plymouth Meeting, USA), and saline was infused into the jugular vein at a rate of 11 µl/min. Baseline glucose was measured 30 and 0 min before the start of the clamp. At time point 0 min, insulin (125 mU/kg/min; Actrapid HM, NovoNordisk) and glucose (50% glucose; variable) infusions were started and maintained for 150 min. Arterial blood samples were collected every 5 min for glucose determination (Breeze2, Bayer AG), with larger samples collected at −30, 85, 100, and 145 min for insulin measurement. At time point 45 min, glucose infusion was either increased to reach the pre-determined physiological post-prandial euglycemic level of 150 mg/dL (EU), or decreased to maintain a hypoglycemic level of approximately 55 mg/dL (HYPO). To test the anorectic effect of amylin under the glycemic clamp paradigm, following maintenance of the target glycemic level for 20 to 25 min, saline or amylin (20 µg/kg; s.c.; 1 mL/kg injection volume) was injected (85 min from the start of the clamp), and pre-weighed chow pellets (9 g) were given to the animals. Remaining food pellets and crumbs were collected from the cage and manually weighed at 30 and 60 min after amylin administration. Insulin was measured in serum in duplicate using Milliplex Map Rat Endocrine kit (RENDO-85 K, Millipore). EU and HYPO clamps were run as two independent experiments in different cohorts of rats.

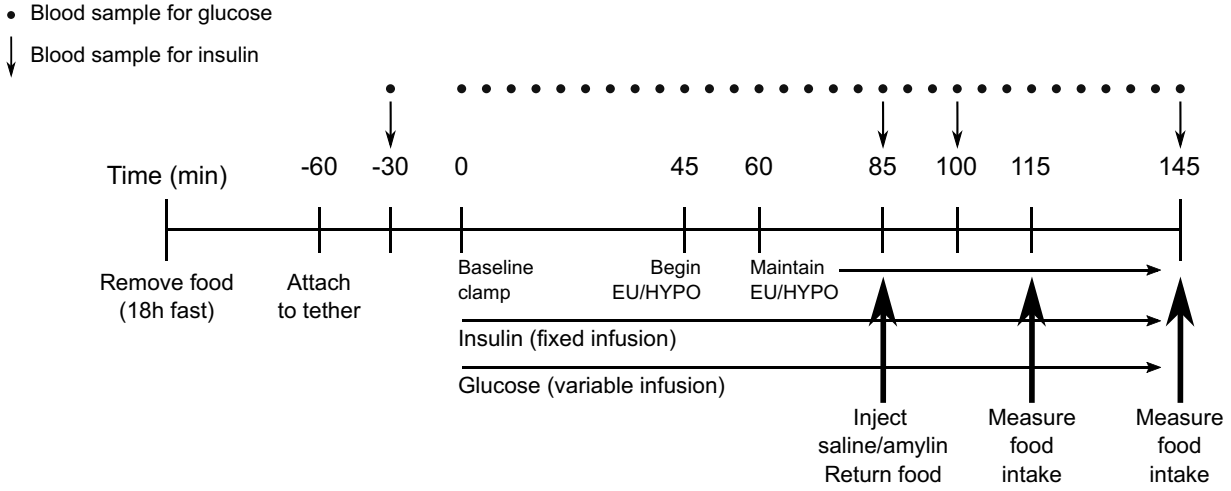

Fig. 2. Experimental timeline for the hyperinsulinemic-euglycemic (EU) and -hypoglycemic (HYPO) clamp experiments. Following an 18-hour fast, male Wistar rats were attached to a weighted swivel tether and saline was infused via the jugular vein catheter for 60 min. At timepoints −30 and 0 min, baseline blood glucose was measured via the carotid artery catheter; blood glucose was then measured every 5 min for the remainder of the clamp. Larger blood samples were collected at −30, 85, 100, and 145 min for insulin level determination. Beginning at timepoint 0 min, insulin (125 mU/kg/min) and glucose (variable infusion rate) were infused into the jugular vein catheter. After 45 min of baseline clamp, over a 15-min period, glucose infusion rates (GIR) were increased to reach target of approximately 150 mg/dL for EU, or reduced to reach the HYPO target of 55 mg/dL. This target level was then maintained for 20–25 min, and at 85 min rats were treated with saline or amylin (20 µg/kg; s.c.) and provided with chow. GIRs were adjusted to maintain the target glycemic levels in freely-feeding rats until 145 min, with 30-min food intake measured at timepoint 115 min, and 60-min food intake measured 145 min. In the pERK experiment, the same procedure was followed, however, following saline or amylin injection at 85 min, food was withheld and rats were deeply anesthetized and perfused 15 min after treatment.

2.5. Influence of glycemic state on amylin-induced phosphorylation of ERK

In a final experiment, the influence of the glycemic state on amylin-induced neuronal signaling in the AP was investigated in a third cohort of rats. Thus, during HYPO or EU clamps, rats received either saline or amylin (20 μg/kg; s.c.), and brain tissue was processed for the detection of pERK using immunohistochemistry. Rats were randomly assigned into one of the following four groups (4–5 rats/group): EU/saline, EU/amylin, HYPO/saline, or HYPO/amylin. The experimental timelines were similar to the previous EU and HYPO clamp experiments (See Fig. 2). Rats received either saline or amylin at 85 min, but unlike in the previous experiments, food was withheld. Fifteen minutes following the injection, the final blood samples were collected for glucose and insulin measurements, and a bolus of pentobarbital (60 mg/kg, i.v.) was administered. Rats were then transcardially perfused with 0.1 M phosphate buffer (PB) followed by 4% PFA in PB. Brains were removed, post-fixed for two hours in 4% PFA in PB, and then transferred to 20% sucrose solution for 36–48 h at 4 °C. Brains were frozen in chilled hexane, and 20-μm coronal brain sections containing the AP were collected using a cryostat (Leica CM3050S) and thaw-mounted onto adhesion glass slides. Brain sections were immunostained using pERK1/2 antibody (1:1000; Cell Signaling Technology #9101), which was visualized using 3,3-diaminobenzidine-tetrahydrochloride (DAB) chromogen and quantified as described previously [6].

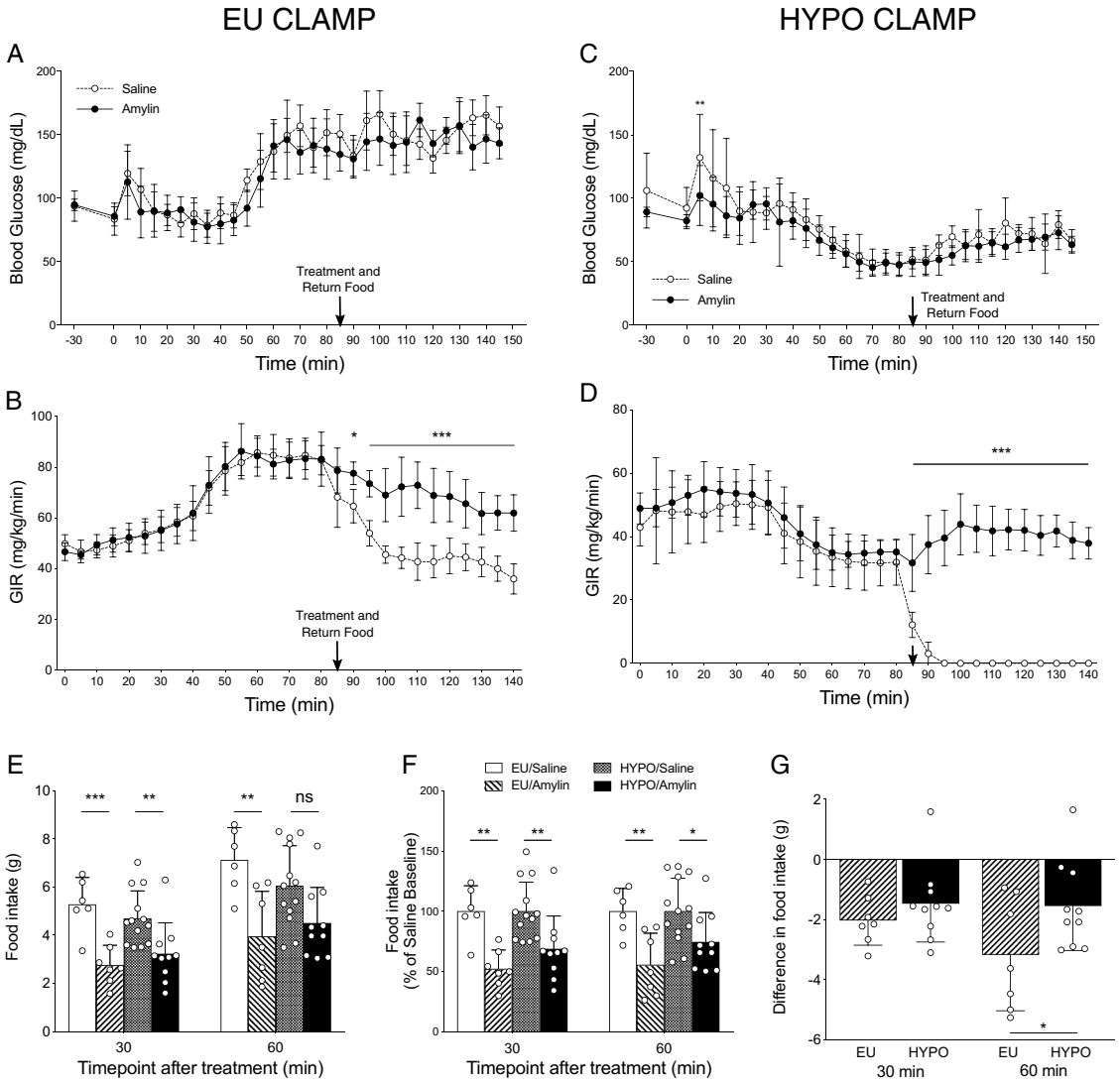

Fig. 3. Effect of amylin on food intake and glucose infusion rate under hyperinsulinemic-euglycemic (EU) and -hypoglycemic (HYPO) clamp conditions. Blood glucose levels (A) from saline- (white circles; $n = 6$) and amylin- (black circles; $n = 7$) treated rats during EU clamps. Basal fasting glucose levels were maintained from 0 to 45 min, at which point, glucose infusion rate (GIR; B) was increased to mimic a postprandial glycemic state between 145 and 155 mg/dL, which was maintained from 60 to 145 min of the clamp. Blood glucose levels (C) from saline- (white circles; $n = 13$) and amylin- (black circles; $n = 10$) treated rats during HYPO clamps. Basal fasting glucose levels were maintained from 0 to 45 min, at which point, GIR (D) was decreased to reach a hypoglycemic state of approximately 55 mg/dL, which was maintained from 60 to 145 min of the clamp. In both EU and HYPO clamps, at 85 min, rats were treated with saline or amylin (20 μg/kg, s.c.) and food was provided. Food intake was measured 30 and 60 min later (E). To directly compare the effectiveness of amylin to reduce food intake under EU and HYPO conditions, food intake data were represented as the percent of saline control food intake (F) and then analyzed using two-way ANOVA for glycemic state (EU or HYPO) and treatment (saline or amylin). The absolute difference in food intake between EU and HYPO amylin-treated rats and their respective saline controls is shown in (G). All data are expressed as mean ± SD; symbols denote significant differences between saline- and amylin-treated groups; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

2.6. Statistical analysis

All data are shown as mean ± SD. Significance of differences between two groups was tested using unpaired t-tests, and data sets with two factors (treatment and glycemic level) were analyzed using two-way ANOVA, followed by Bonferroni's multiple comparison when appropriate (GraphPad Prism 9 for Mac). A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Amylin-induced reduction in food intake is preserved during hyperinsulinemic/euglycemic clamp

To determine whether the glycemic state influences the ability of exogenous amylin to reduce food intake or buffer meal-induced glucose excursions in the blood, rats were first clamped at a euglycemic blood glucose level corresponding to the postprandial state (approximately 150 mg/dL; Fig. 3A). After achieving a steady-state, peripheral amylin (20 μg/kg) or vehicle was administered and rats were provided access of food. Similar to what was observed in our pilot experiments, immediately following meal initiation, vehicle-treated rats required a significantly greater reduction in GIR to maintain the clamp at 150 mg/dL glucose, compared to the reduction required by the amylin-treated group (Fig. 3B); we observed a main effect of treatment ($F\ [1,319] = 143.1$, $P<0.001$), and detected individual differences between saline- and amylin-treated rats beginning at 85 min ($P<0.05$). This difference was more pronounced between 90 and 140 min ($P<0.001$), where amylin-treated rats required 1.5 to 2 times more exogenous glucose than saline-treated rats to maintain the EU clamp. Amylin treatment significantly reduced food intake measured at 30 ($P<0.001$) and 60 min ($P<0.01$) compared to vehicle (Fig. 3E; white and lined bars). By first demonstrating that amylin's effect on food intake is intact during the euglycemic clamp, we can conclude that the hyperinsulinemic clamp protocol itself does not influence amylin's ability to reduce food intake.

3.2. Amylin-induced reduction in food intake is blunted during hyperinsulinemic/hypoglycemic clamp

Next, we investigated if hypoglycemia would interfere with amylin-induced reduction in food intake. Rats were clamped at a hypoglycemic level that still permitted the fine-motor coordination required to eat (55 mg/dL, Fig. 3C). When steady-state was achieved, peripheral amylin or vehicle was administered and all rats began eating when given access to food. In order to maintain HYPO in rats eating a meal, the GIR in vehicle-treated rats was lowered prior to the first post-ingestion/injection blood sample at minute 85, and was turned to 0 mg/kg/min by minute 95 (Fig. 3D). In contrast, the amylin-treated rats required an increase in GIR from the pre-prandial/pre-injection state, resulting in a main effect of amylin treatment ($F\ [1,591] = 945.9$, $P<0.001$), with significant increases in GIR between minutes 85 and 140 compared to the vehicle group ($P<0.001$). Amylin treatment in HYPO rats significantly reduced food intake at 30 min after treatment ($P<0.01$), but not 60, compared to vehicle (Fig. 3E; hatched and black bars).

3.3. Comparison of food intake and circulating insulin levels during euglycemic and hypoglycemic clamps

As a primary objective of these experiments was to determine if hypoglycemia alters the ability of acute amylin administration to reduce eating, food intake data obtained from the EU and HYPO clamps were further analyzed in relation to one another. First, using two-way ANOVA, the main effects of amylin treatment and glycemic state were analyzed (Fig. 3E). A main effect of treatment ($F\ [1,32] = 25.86$, $P<0.001$), but not glycemic state, was detected in the 30 min data, with a significant difference between saline and amylin observed in both EU ($P<0.001$) and HYPO ($P<0.01$) states. At 60 min, a main effect of treatment ($F\ [1,32] = 17.68$, $P<0.001$), but not glycemic state, was again observed, however a significant reduction of amylin was only observed in the EU ($P<0.01$) and not the HYPO state. To account for slight differences in baseline food intake between the EU and HYPO clamps, food intake data were also represented as a percentage of each group's saline control (Fig. 3F). As observed in the raw data, a main effect of treatment was observed at 30 and 60 min ($F\ [1, 32] = 23.66$ and 15.88, $P<0.001$ for both). At 30 min, amylin reduced food intake in both EU and HYPO states ($P<0.01$), though the effect of amylin was less significant in HYPO rats 60 min after administration ($P<0.01$ for EU, $P<0.05$ for HYPO). Further, when we calculated the absolute reduction in food intake versus the saline control (Fig. 3G), amylin treatment reduced 60-min food intake by an average of 3.17 ± 1.86 g (55.5% of saline controls) in the EU state, which was significantly more than the reduction of 1.58 ± 1.48 g (74.4% of saline controls) in the HYPO state ($t\ [15] = 1.99$, $P<0.05$). Thus, even though amylin was able to reduce food intake under this specific HYPO condition, the effect was attenuated compared to EU.

Insulin was measured at timepoints −30, 85, 100, and 145 min during each clamp, as this could also influence GIR and food intake. However, regardless of treatment or target glycemic state, insulin levels were not different across groups at any timepoint during the clamp procedure (Table 1), confirming the validity of the clamp conditions with fixed insulin infusion rates.

3.4. Glycemic state does not influence amylin-induced pERK in the area postrema

Previous work from our group demonstrated that amylin induces the phosphorylation of the signaling molecule ERK in the AP, and that blockade of this signaling pathway reduces amylin's effect on food intake [6]. Here we asked whether hypoglycemia influences the amylin-induced phosphorylation of ERK in the AP. Fig. 4A shows the quantification of the number of neurons positive for pERK in the AP collected from rats 15 min after saline or amylin (20 μg/kg) treatment, and maintained in the EU or HYPO state. Two-way ANOVA detected a main effect of amylin treatment on the number of pERK-positive cells ($F\ [1,15] = 17.79$, $P<0.001$); however, no effect of the glycemic state was observed. Multiple comparisons demonstrated that amylin induced a significant pERK response in the AP of both EU ($P<0.05$) and HYPO ($p<0.01$) rats. Representative photomicrographs of the AP from HYPO rats treated with saline or amylin are shown in Fig. 4B and C.

4. Discussion

Advancements in insulin therapy to treat type 1 and type 2 diabetes have achieved tremendous benefits in controlling basal and postprandial blood glucose levels by using different insulin administration methods and combination therapies. In fact, the combination of insulin and amylin resulted in a better control of long-term body weight and of postprandial glucose excursion compared to insulin monotherapy [12, 13]. Despite these advances, hypoglycemia remains a serious side effect of insulin therapy. Therefore, and because of the success of insulin and amylin combination therapy, more research needs to be done to

Table 1
Mean ± SD arterial insulin levels (ng/mL) measured during EU and HYPO glucose clamps.

	EU/Saline		EU/Amylin		HYPO/Saline		HYPO/Amylin	
Time (min)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
−30	0.48	0.32	0.76	0.85	0.28	0.18	0.56	0.37
85	3.19	0.31	4.03	0.39	3.29	0.63	3.90	0.99
100	3.04	0.21	3.95	0.22	3.79	0.66	3.76	0.70
145	3.41	0.34	4.04	0.36	4.28	1.32	3.99	0.54

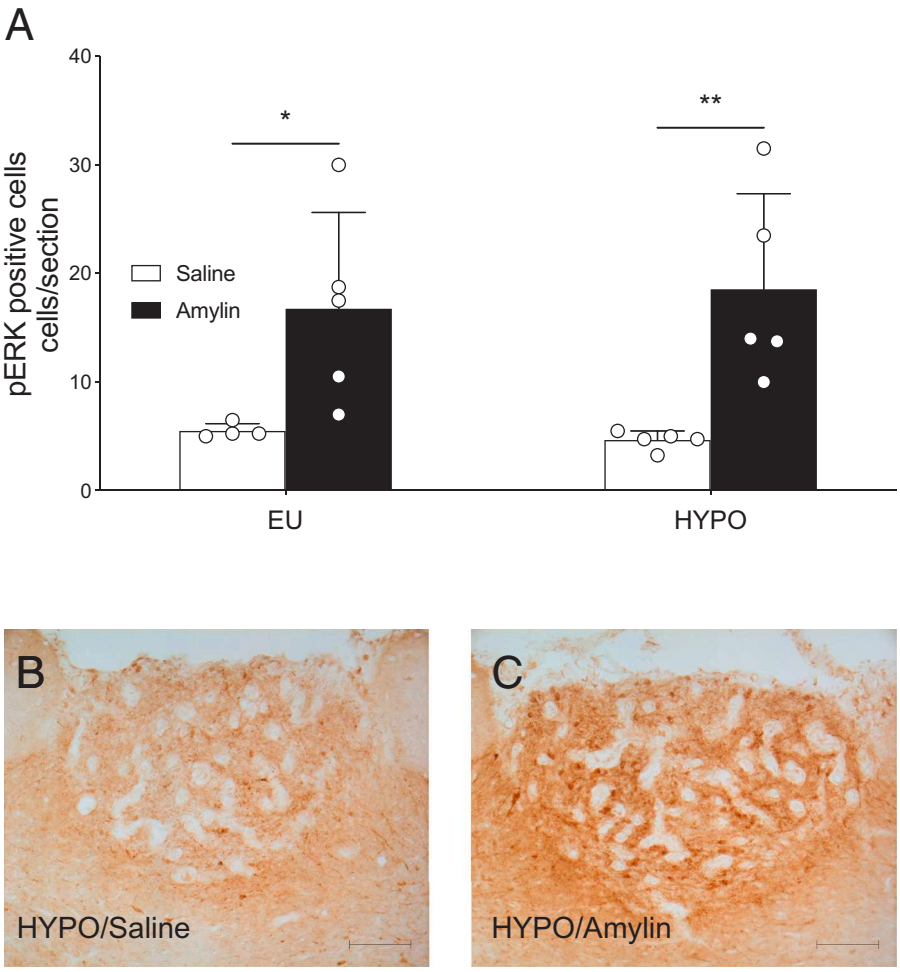

Fig. 4. Amylin treatment leads to the phosphorylation of ERK in the area postrema under euglycemic and hypoglycemic conditions. Mean ± SD number of pERK-positive cells per section of AP quantified is shown (A) for rats maintained at a euglycemic (EU) level and treated with saline (white bar, $n = 4$) or amylin (black bar, $n = 5$), or maintained at a hypoglycemic (HYPO) level and treated with saline (white bar, $n = 5$) or amylin (black bar, $n = 5$). Representative photomicrographs are shown from the hypoglycemic/saline group (B) and the hypoglycemic/amylin (C) group. Data are expressed as mean ± SD; symbols denote significant differences between saline- and amylin-treated groups; $^{*}p<0.05$, $^{**}p<0.01$.

investigate the function of amylin under such conditions. In this study, we investigated the efficacy of amylin under hypoglycemic conditions. In particular, we asked whether hypoglycemia limits the acute, food intake-reducing effect of amylin to prevent a shortage of nutrient supply. To answer this question, we performed hyperinsulinemic-euglycemic and -hypoglycemic clamps in male Wistar rats. The results of these studies reveal three main findings: 1) Hypoglycemia attenuates the acute food intake-reducing effect of amylin. 2) Following amylin treatment, increased GIR is required to maintain glycemic levels compared to controls rats, both under EU and HYPO conditions. 3) The amylin-induced phosphorylation of ERK in the AP is not affected by hypoglycemia.

4.1. Hypoglycemia attenuates acute amylin-induced inhibition of food intake

To test our hypothesis that hypoglycemia may influence the effectiveness of amylin to reduce eating, we set out to develop a new glycemic clamp paradigm which allowed freely-moving rats to eat while also maintaining a constant glycemic level via hyperinsulinemic clamp infusions. Upon establishing the clamp protocol, we first determined that the hyperinsulinemic clamp itself does not influence amylin action by performing the experiment under euglycemia conditions. The eating inhibitory effect of amylin in rats subjected to the EU clamp was consistent with the well-known function of amylin to significantly reduce food intake [14,15]. These results therefore confirmed that neither the clamp nor the hyperinsulinemic state had an effect on amylin's action.

When we performed a hyperinsulinemic HYPO clamp, we observed that the acute amylin-induced reduction in food intake was attenuated. While food intake following amylin was similarly reduced during the first 30 min in both glycemic states, the potency of amylin to reduce eating was lessened at the 60 min timepoint during hypoglycemia. We did not, however, observe a complete blockade of amylin's inhibitory effect on eating, which had been previously shown for amylin's effect on gastric emptying under hypoglycemic conditions [3-5]. As discussed below, this disparity likely reflects the different levels of hypoglycemia achieved in these studies.

4.2. Amylin treatment results in higher GIR in both EU and HYPO clamps

Our study clearly demonstrates that when amylin is administered immediately prior to a meal, the appearance of prandial blood glucose is dramatically reduced, as is evidenced by the increased GIR necessary to keep amylin-treated rats at the same target glycemic level as vehicle-treated rats. The impact of amylin on glucose was observed within minutes after amylin injection, and it is therefore unlikely to be a direct consequence of the reduction in food intake measured at 30 and 60 min. While it was not systematically quantified, we observed no differences in the short latency to begin eating when food was made available between saline- and amylin-treated rats, which had been fasted for 18 h. Over the course of our pilot and primary experiments, we observed that in order to tightly maintain the clamped concentration in freely-feeding rats, GIR must be lowered already prior to the first blood sample after meal onset in the vehicle-treated groups. This was not the case in rats treated with amylin, especially during the hypoglycemic clamp in which it was

actually necessary to increase GIR above the level before the rats started to eat. This is consistent with delayed glucose excursions observed in volunteers that were either healthy or suffering from Type 1 diabetes after treatment with the amylin analogue pramlintide [16, 17]. One caveat of these studies was that 50% less insulin was administered on the test days when pramlintide was given (per standard FDA recommendations to prevent hypoglycemia [18]), raising the question if lower insulin levels contributed to the reduced glucose excursions [16]. In the present study, however, insulin infusions were steady across all clamps, and insulin measured in the blood was not different in any condition, thus demonstrating that the effect of amylin to delay glucose appearance occurs even when insulin levels are matched.

Because amylin both increased GIR and reduced eating in rats maintained in either a HYPO or EU state, we can conclude that this level of hypoglycemia (between 55 and 60 mg/dL) does not completely block the ability of amylin to limit blood glucose appearance or reduce food intake. Amylin was less effective at reducing food intake in the HYPO rats, but there still was an observable and significant effect on eating. The idea of a hypoglycemic brake on amylin function was based on data showing that amylin failed to reduce gastric emptying or suppress glucagon release under hypoglycemic conditions [3, 19, 20]. A key factor mediating this effect, however, was the induction of extreme hypoglycemic states; at 2.5 mmol/l (45 mg/dL), approximately 95% of amylin action on gastric emptying was suppressed. At a glycemic level more comparable to that used in the present study (55 mg/dL), extrapolation from the dose-response curve indicated that the reduction in the suppression of gastric emptying was ~50%, which is similar to the observed reduction in amylin's eating-inhibitory effect [3]. Based on our pilot studies, in which we observed that rats clamped lower than 55 mg/dL would begin showing fine motor deficits that could influence their ability to eat, we opted to target a glucose level above this threshold. Collectively, these data refine the idea of a hypoglycemic brake on amylin action, demonstrating that, while attenuated, amylin retains some capacity to reduce eating and limit the appearance of glucose in the blood under the hypoglycemic conditions tested.

4.3. Amylin-induced phosphorylation of ERK in the AP is not affected by hypoglycemia

The AP is not only an important brain area in amylin sensing, but can also sense changes in blood glucose levels [21]. This co-sensitivity has been hypothesized to play an important functional role during hypoglycemia [9]. AP neurons that are excited by amylin show reduced spontaneous in vitro activity at hypoglycemic levels; the lower the glucose, the lower neuronal activity [9]. In the present study, we showed that low glucose levels attenuate amylin's effect on food intake, suggesting that a normal or elevated plasma glucose level is a necessary precondition for the full satiating effect of amylin to occur. As the mechanisms influencing this difference are still unclear, we tested the hypothesis that euglycemic levels are permissive of amylin-induced phosphorylation of ERK in the AP. We previously showed that peripheral amylin treatment induces pERK in the AP, with peak expression of pERK detected at 15 min after treatment [6]. We also found that pERK was functionally relevant for amylin action, as blockade of ERK phosphorylation with the MEK inhibitor U0126 in the fourth ventricle blunted the food intake-reducing effect of peripheral amylin under certain conditions [6]. We anticipated that the attenuation of amylin's eating inhibitory effect under hypoglycemic conditions would correlate with a decreased number of pERK-positive neurons after amylin treatment in the HYPO rats compared to EU. We observed, however, that the number of pERK-positive cells per AP section was similar in the two glycemic states; this was true for saline as well as for amylin-injected rats. Consistent with our previous findings [6], amylin significantly increased pERK levels compared to saline after 15 min, and this was true in both EU and HYPO states.

Hence, we have seen an attenuated food intake-reducing response of amylin during hypoglycemia, but no change in the pERK formation in the AP. As we showed previously that ERK phosphorylation plays a role in amylin action [6], it seems that the effect of hypoglycemia on amylin's satiating response is located downstream or is independent of the ERK phosphorylation. We have observed a similar dichotomy in rats lacking function leptin receptors—the satiating effect of amylin was reduced in the absence of the leptin receptor, but neurons in the AP still mounted a normal Fos response following amylin treatment [10]. In both cases, it seems that amylin is still capable of activating AP neurons, but the ability of hypoglycemia (present study) or loss of leptin signaling [10] to limit amylin-induced reduction of food intake is located downstream of the activation of either pERK or Fos, respectively, in the AP. The lateral parabrachial nucleus, for example, is a brain nucleus that is both targeted by amylin-activated AP neurons [22, 23], and sensitive to changes to glucose levels [24], therefore representing a potential secondary site of convergence for amylin and glucose signaling. It is also possible that the mere presence of pERK in the AP tells us little about how it is altering cell function. Interestingly, a recent study delineated a link between neuronal activity pattern and subsequent changes in gene expression profile, which is mediated by MAPK/ERK signaling pathways [25], such that brief or sustained neuronal activity led to different patterns of gene expression, though both were dependent on MAPK/ERK signaling. Based on data showing that glucose levels can alter firing rates of amylin-sensitive neurons [9], it is possible that low glucose levels can modulate the activity pattern of amylin-activated cells, which has the potential to alter the downstream consequences of phosphorylated ERK without altering the presence of amylin-induced pERK.

4.4. Future directions and conclusion

Perhaps the most striking finding from this study was the dramatic increase in GIR necessary to offset amylin's stark reduction of the appearance of prandial blood glucose, and the fact that this was observed in both euglycemic and hypoglycemic conditions. One plausible explanation for these findings is that amylin's suppression of meal-stimulated glucagon secretion contributes greatly to this outcome. Glucagon helps to maintain glycemia at a healthy level by promoting glycogenolysis [26] and gluconeogenesis [27], and its release is stimulated by both hypoglycemia and in response to nutrients [20]. Thus, due to the suppression of nutrient-stimulated glucagon secretion by amylin treatment, more exogenous glucose was required to maintain the targeted level of glycemia. What remains to be understood, is why the difference in GIR between saline- and amylin-treated rats was even greater during the HYPO clamp than during the EU (a difference of approximately 40 mg/kg/min glucose during HYPO versus 20 mg/kg/min during EU). Based on the evidence that hypoglycemia limits amylin's ability to suppress glucagon [5], we would have hypothesized a smaller difference in GIR during the HYPO clamp; due to the hypoglycemic brake on amylin action, glucagon's counterregulation of the hypoglycemia should still be intact. There is evidence that counterregulatory hormones, such as norepinephrine, epinephrine and glucagon, were similar in humans receiving pramlintide or placebo during hypoglycemia [28]. However, future studies are needed to compare levels of glucagon to confirm the role of glucagon in the observed GIR effect, and whether this role varies under HYPO and EU conditions. Additionally, use of isotopic glucose tracer would be a valuable way to determine how quickly glucose is absorbed from the carbohydrates in the food, to determine the precise contribution of endogenous and exogenous glucose to the blood glucose levels measured during the clamps, and to understand how amylin and glycemic state influence these processes.

In conclusion, the data presented here add several insights into the posited brake that hypoglycemia places on amylin action. For more than two decades, it has been known that under hypoglycemic conditions amylin's ability to suppress glucagon release and slow gastric emptying are reduced. Here we demonstrate using a novel glycemic clamp

paradigm that hypoglycemia also attenuates the food intake-reducing effect of amylin in rats. We also showed that amylin quickly limits prandial blood glucose appearance, as evidenced by the pre-emptive and sustained increases in GIRs required to clamp blood glucose at either a hypoglycemic or euglycemic state following amylin treatment at meal onset. Finally, we showed hypoglycemia does not influence amylin's ability to activate the ERK signaling pathway in AP neurons.

Declaration of Competing Interest

Authors declare the following potential conflict of interest. TAL has received research support from Novo Nordisk and Boehringer Ingelheim; the supported projects are unrelated to the research shown here. The remaining authors have no potential conflicts of interest.

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References

[1] A. Young, Amylin and the integrated control of nutrient influx. Advances in Pharmacology, Academic Press, 2005, pp. 67–77.
[2] D.L. Hay, S. Chen, T.A. Lutz, D.G. Parkes, J.D. Roth, Amylin: pharmacology, physiology, and clinical potential, Pharmacol Rev 67 (3) (2015) 564–600.
[3] B.R. Gedulin, A.A. Young, Hypoglycemia overrides amylin-mediated regulation of gastric emptying in rats, Diabetes 47 (1) (1998) 93–97.
[4] B.R. Gedulin, C.M. Jodka, K. Herrmann, A.A. Young, Role of endogenous amylin in glucagon secretion and gastric emptying in rats demonstrated with the selective antagonist, AC187, Regul. Pept. 137 (3) (2006) 121–127.
[5] B.R. Gedulin, T.J. Rink, A.A. Young, Dose-response for glucagonostatic effect of amylin in rats, Metabolism 46 (1) (1997) 67–70.
[6] C.S. Potes, C.N. Boyle, P.J. Wookey, T. Riediger, T.A. Lutz, Involvement of the extracellular signal-regulated kinase 1/2 signaling pathway in amylin's eating inhibitory effect, Am J Physiol Regul Integr Comp Physiol 302 (3) (2012) R340–R351.
[7] F.E. Braegger, L. Asarian, K. Dahl, T.A. Lutz, C.N. Boyle, The role of the area postrema in the anorectic effects of amylin and salmon calcitonin: behavioral and neuronal phenotyping, Eur J Neurosci 40 (7) (2014) 3055–3066.
[8] G.L. Edwards, B.R. Gedulin, J. C., R.P. Dilts, M. C.C., A. Young, Area postrem (AP)-lesions block the regulation of gastric emptying by amylin, Neurogastroenterol Motil 10 (1998) 26.
[9] T. Riediger, H.A. Schmid, T.A. Lutz, E. Simon, Amylin and glucose co-activate area postrema neurons of the rat, Neurosci Lett 328 (2) (2002) 121–124.
[10] S. Duffy, T.A. Lutz, C.N. Boyle, Rodent models of leptin receptor deficiency are less sensitive to amylin, Am J Physiol Regul Integr Comp Physiol 315 (4) (2018) R856–R865.
[11] M. Bohland, A.V. Matveyenko, M. Saberi, A.M. Khan, A.G. Watts, C.M. Donovan, Activation of hindbrain neurons is mediated by portal-mesenteric vein glucosensors during slow-onset hypoglycemia, Diabetes 63 (8) (2014) 2866–2875.
[12] D.G. Maggs, M. Fineman, J. Kornstein, T. Burrell, S. Schwartz, Y. Wang, J. A. Ruggles, O.G. Kolterman, C. Weyer, Pramlintide reduces postprandial glucose excursions when added to insulin lispro in subjects with type 2 diabetes: a dose-timing study, Diabetes Metab Res Rev 20 (1) (2004) 55–60.
[13] C. Weyer, D.G. Maggs, A.A. Young, O.G. Kolterman, Amylin replacement with pramlintide as an adjunct to insulin therapy in type 1 and type 2 diabetes mellitus: a physiological approach toward improved metabolic control, Curr Pharm Des 7 (14) (2001) 1353–1373.
[14] T.A. Lutz, N. Geary, M.M. Szabady, E. Del Prete, E. Scharrer, Amylin decreases meal size in rats, Physiol Behav 58 (6) (1995) 1197–1202.
[15] U. Arnelo, J. Permert, T.E. Adrian, J. Larsson, P. Westermark, R.D. Reidelberger, Chronic infusion of islet amyloid polypeptide causes anorexia in rats, Am J Physiol 271 (6 Pt 2) (1996) R1654–R1659.
[16] L. Hinshaw, M. Schiavon, A. Mallad, C.D. Man, R. Basu, A.E. Bharucha, C. Cobelli, R.E. Carter, A. Basu, Y.C. Kudva, Effects of delayed gastric emptying on postprandial glucose kinetics, insulin sensitivity, and beta-cell function, Am J Physiol Endocrinol Metab 307 (6) (2014) E494–E502.
[17] L. Hinshaw, M. Schiavon, V. Dadlani, A. Mallad, C. Dalla Man, A. Bharucha, R. Basu, J.R. Geske, R.E. Carter, C. Cobelli, A. Basu, Y.C. Kudva, Effect of pramlintide on postprandial glucose fluxes in type 1 diabetes, J Clin Endocrinol Metab 101 (5) (2016) 1954–1962.
[18] G.J. Ryan, L.J. Jobe, R. Martin, Pramlintide in the treatment of type 1 and type 2 diabetes mellitus, Clin Ther 27 (10) (2005) 1500–1512.
[19] A. Young, Role of amylin in nutrient intake - animal studies, Diabet Med 14 (Suppl 2) (1997) S14–S18.
[20] A. Young, Inhibition of glucagon secretion, Adv Pharmacol 52 (2005) 151–171.
[21] A. Adachi, M. Kobashi, M. Funahashi, Glucose-responsive neurons in the brainstem, Obes Res 3 (5) (1995) 735S–740S. **Suppl**.
[22] C.S. Potes, T.A. Lutz, T. Riediger, Identification of central projections from amylin-activated neurons to the lateral hypothalamus, Brain Res 1334 (2010) 31–44.
[23] L. Boccia, C.Le Foll, T.A. Lutz, Noradrenaline signaling in the LPBN mediates amylin's and salmon calcitonin's hypophagic effect in male rats, FASEB J 34 (11) (2020) 15448–15461.
[24] A.S. Garfield, B.P. Shah, J.C. Madara, L.K. Burke, C.M. Patterson, J. Flak, R.L. Neve, M.L. Evans, B.B. Lowell, M.G. Myers, Jr., L.K. Heisler, A parabrachial-hypothalamic cholecystokinin neurocircuit controls counterregulatory responses to hypoglycemia, Cell Metab 20 (6) (2014) 1030–1037.
[25] K.M. Tyssowski, N.R. DeStefino, J.H. Cho, C.J. Dunn, R.G. Poston, C.E. Carty, R. D. Jones, S.M. Chang, P. Romeo, M.K. Wurzelmann, J.M. Ward, M.L. Andermann, R.N. Saha, S.M. Dudek, J.M. Gray, Different Neuronal Activity Patterns Induce Different Gene Expression Programs, Neuron, 98 (3) (2018) 530–546, e11.
[26] C.J. Ramnanan, D.S. Edgerton, G. Kraft, A.D. Cherrington, Physiologic action of glucagon on liver glucose metabolism, Diabetes Obes Metab 13 (1) (2011) 118–125. **Suppl**.
[27] A. Gastaldelli, E. Toschi, M. Pettiti, S. Frascerra, A. Quinones-Galvan, A.M. Sironi, A. Natali, E. Ferrannini, Effect of physiological hyperinsulinemia on gluconeogenesis in nondiabetic subjects and in type 2 diabetic patients, Diabetes 50 (8) (2001) 1807–1812.
[28] T. Heise, L. Heinemann, S. Heller, C. Weyer, Y. Wang, S. Strobel, O. Kolterman, D. Maggs, Effect of pramlintide on symptom, catecholamine, and glucagon responses to hypoglycemia in healthy subjects, Metabolism 53 (9) (2004) 1227–1232.